Proteomic Analysis of the *Plasmodium berghei* Gametocyte Egressome and Vesicular bioID of Osmiophilic Body Proteins Identifies Merozoite TRAP-like Protein (MTRAP) as an Essential Factor for Parasite Transmission*[S]

Jessica Kehrer‡, Friedrich Frischknecht‡, and Gunnar R. Mair‡§

Malaria transmission from an infected host to the mosquito vector requires the uptake of intraerythrocytic sexual precursor cells into the mosquito midgut. For the release of mature extracellular gametes two membrane barriers—the parasite parasitophorous vacuole membrane and the host red blood cell membrane—need to be dissolved. Membrane lysis occurs after the release of proteins from specialized secretory vesicles including osmiophilic bodies. In this study we conducted proteomic analyses of the *P. berghei* gametocyte egressome and developed a vesicular bioID approach to identify hitherto unknown proteins with a potential function in gametocyte egress. This first *Plasmodium* gametocyte egressome includes the proteins released by the parasite during the lysis of the parasitophorous vacuole membrane and red blood cell membrane. BioID of the osmiophilic body protein MDV1/PEG3 revealed a vesicular proteome of these gametocyte-specific secretory vesicles. Fluorescent protein tagging and gene deletion approaches were employed to validate and identify a set of novel factors essential for this lysis and egress process. Our study provides the first *in vivo* bioID for a rodent malaria parasite and together with the first *Plasmodium* gametocyte egressome identifies MTRAP as a novel factor essential for mosquito transmission. Our data provide an important resource for proteins potentially involved in a key step of gametogenesis. *Molecular & Cellular Proteomics 15: 10.1074/mcp.M116.058263, 2852–2862, 2016.*

Malaria caused 214 million clinical cases in 2015 (1). The infection is initiated when the mosquito injects sporozoites from its salivary glands into the skin during a bite. Salivary gland sporozoites are the result of a three-week developmental process that started with the uptake of sexual precursor cells during a previous blood meal by the female mosquito. The uptake of these intraerythrocytic, sexual precursor cells (so-called gametocytes) can lead to infection of the *Anopheles* vector. To establish an infection in the mosquito, male and female gametocytes must first differentiate into mature gametes and egress from the red blood cell; only then can they participate in fertilization and zygote formation, and develop further into ookinetes that will cross the midgut epithelium to form oocysts in which sporozoites are formed. Gametogenesis (the process of generating free gametes from intracellular precursors) requires the change of key environmental parameters during the blood meal: this includes a drop in temperature from 37 °C to around 20 °C, an increase of pH and the presence of xanthurenic acid from the mosquito (2–4). The egress of fully differentiated gametes finally necessitates the lysis of two membrane barriers: the outer red blood cell membrane (RBCM)[1] of the host and the inner parasitophorous vacuole membrane (PVM) maintained by the parasite. The sequential, inside-out lysis of PVM and RBCM occurs in response to the exocytosis of specialized secretory vesicles only present in the gametocyte including the so-called osmiophilic bodies (OB). Only four proteins involved in that process are known to be secreted. PPL2, GEST, G377, and MDV1/PEG3. Although MDV1/PEG3, GEST, and G377 localize to OB, PPLP2 has been suggested to inhabit a separate population of egress vesicles. In *P. falciparum*, this perforin-like protein is expressed in male and female gametocytes alike and released in a calcium-dependent manner upon activation (5). *pplp2* null mutants fail to rupture the RBC membrane but are still able to lyse the PVM. In *P. berghei* it is important for male exflagellation whereas females remain viable and are able to produce zygotes with fertile males (6). GEST and MDV1/PEG3 have been shown to colocalize in OB

From the ‡Integrative Parasitology, Center for Infectious Diseases, University of Heidelberg Medical School, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany

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[1] The abbreviations used are: RBCM, red blood cell membrane; RBC, red blood cell; PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane OB, osmiophilic bodies; i.p., intraperitoneal; i.v., intravenous; ER, endoplasmic reticulum; IFA, immuno fluorescence assay; GFP, green fluorescent protein.

(7) whereas MDV1/PEG3 staining remains unaltered in Δ*gest* parasites, indicating that GEST does not influence the formation of OB or the packaging of MDV1/PEG3 to OB. GEST is expressed in females and to a lesser degree in males. Δ*gest* parasites are still able to lyse the PVM but are not able to egress from the RBC. Furthermore male Δ*gest* microgametes show aberrant exflagellation (7). Δ*mdv1/peg3* parasites in contrast have no or few OB and are defect in PVM and RBC rupture, which leads to low transmission into mosquitoes (8). Δ*g377* parasites finally have a reduced number of OB but no defect in MDV1/PEG3 packaging (9); microgametes still exflagellate like wildtype but egress of females is significantly reduced, which results in reduced transmission to the mosquito (10). Each of these four proteins is important for efficient gametocyte egress, but not strictly essential. They share no amino acid sequence similarities. Beyond this, no other protein components are known to be involved in egress.

Here we provide a dual approach for the identification of novel egress components in the rodent malaria parasite *P. berghei*: mass spectrometric analysis of egress supernatants and bioID of osmiophilic bodies. For bioID we fused the mutated biotin ligase BirA* from *Escherichia coli* (11) to MDV1/PEG3. We develop and present the first bioID approach for the *in vivo* biotin labeling of parasite proteins while present in the circulatory system of the rodent host. Based on these methods we identify novel proteins in *P. berghei* gametocytes possibly involved in gamete egress; using fluorescent protein tagging and gene knockout we validate gametocyte OB localization of selected candidates and identify MTRAP as an essential, novel egress factor.

EXPERIMENTAL PROCEDURES

Animals—All animal work was performed according to European regulations in compliance with FELASA guidelines and regulations, and approved by the state authorities (Regierungspräsidium Karlsruhe).

Parasite Transfection—Transfection of respective schizonts was done by electroporation using the Amaxa nucleofector kit, followed by selection of mutant parasites with pyrimethamine supplemented in drinking water and limiting dilution of clonal lines as described (12, 13). Genotyping by PCR was performed as shown in Supplemental Figs.

Generation of G377::mCherry Parasites—The 3′ end 1470 bps of the G377 (PBANKA_146300) open reading frame (ORF) were PCR-amplified with primers g3092 and g3093 (primer sequence are available on request), digested with PacI and BamHI and ligated in frame with mCherry into a plasmid containing the human DHFR selection marker. Prior to transfection plasmid pLIS0244 was linearized with BstZ17I.

Generation of mdv1/peg3::bira::myc Parasites*—The 3′ end 661 bps of the MDV1/PEG3 (PBANKA_143220) ORF were PCR-amplified as 2 adjacent products with primers g3094 and g3095, and g3096 and g3097, digested with EcoRI, ligated, and re-amplified with primers g3094 and g3096, thus introducing a unique EcoRI-site in the middle of the amplicon. After digestion with SspI and BamHI the PCR product was ligated in frame with BirA*-cmyc into a plasmid containing the human DHFR selection marker. Prior to transfection the plasmid pLIS0297 was linearized with EcoRI.

Fluorescent Protein Tagging—The 3′ end of the ORF of the following genes were PCR-amplified with primers listed in brackets (a third and fourth primer indicate that individual PCR products were generated and ligated to introduce restriction sites to be used for linearization prior to transfection); restriction enzymes used in cloning are shown next, followed by plasmid name, and enzyme used for linearization: PBANKA_143730 (g3248, g3256, g3249, ScaI, NotI, pLIS0443, EcoRI) PBANKA_091170 (g3236, g3237, EcoRV, BamHI, pLIS0442, EcoRI), PBANKA_144900 (g3148, g3149, SwaI, BamHI, pLIS0371, AflII), PBANKA_136550 (g3171, g3272, MunI, BamHI, pLIS0387, EcoRI), PBANKA_0712600 (g0589, g0590, EcoRI, BamHI, pLIS0356, HindIII), PBANKA_030060 (g3157, g3158, g3159, g3160, EcoIRI, BamHI, pLIS0370, ClaI), PBANKA_132730 (g3171, g3172, EcoRI, BamHI, pLIS0360, HindIII), PBANKA_051280 (g3220, g3221, MunI, BamHI, pLIS0416, BsaBI), PBANKA_103730 (g3242, g3243, XmnI, BamHI, pLIS0425, HindIII), PBANKA_081300 (g3224, g3225, blunt, BamHI, pLIS0418, SwaI), PBANKA_103520 (g3240, g3241, BstZ17I, BamHI, pLIS0424, BsmI), PBANKA_131270 (g3103, g3104, BstZ17I, BamHI, pLIS0281, EcoRI), PBANKA_131270 (g1031, g1032, BstZ17I, BamHI, pLIS0243, BsmI), PBANKA_143220 (g3094, g3095, g3096, g3097, SspI, BamHI, pLIS0297, EcoRI).

MTRAP Knockout—Primers g3349 and g3350, and primers g3321 and g3322 were used to amplify PCR products that were cloned either side of the *Toxoplasma gondii* DHFR/TS selection cassette. Plasmid pLIS0480 was linearized with KpnI and BamHI prior to transfection, and resulted in a deletion of the 712-most bp of the 5′ end of the ORF.

PBANKA_030060 Knockout—Primers g3289 and g3290, and primers g3159 and g3160 were used to amplify PCR products that were cloned either side of the *Toxoplasma gondii* DHFR/TS selection cassette. Plasmid pLIS0450 was linearized with KpnI and BamHI prior to transfection, and resulted in a deletion of the 1870-most bp of the 5′ end of the ORF.

PBANKA_144900 Knockout—Primers g3330 and g3331, and primers g3148 and g3149 were used to amplify PCR products that were cloned either side of the *Toxoplasma gondii* DHFR/TS selection cassette. Plasmid pLIS0457 was linearized with KpnI and BamHI prior to transfection, and resulted in a deletion of the 740-most bp of the 5′ end of the ORF.

Purification and Activation of Gametocytes—Blood of an infected mouse was harvested by cardiac puncture and gametocytes purified using a 49% Nycodenz cushion at 37 °C (1500 rpm; 20 min). Cells were washed once with PBS and resuspended in 30 μl ookinete medium (250 ml RPMI, HEPES, glutamine 12.5 mg Hypoxanthine, 2.5 ml Pen/Strep, 0.5g $NaHCO_3$, 5.12 mg Xanthurenic) at 19 °C for 20min. Supernatants were separated from parasite pellets by a 13000 rpm spin and used for Western blot analyses and mass-spectrometry. To collect supernatants from non-activated cells, gametocytes were kept at 37 °C, resuspended in PBS and the RBC membrane selectively lysed with 3 μl of 0.35% (final concentration of 0.035%).

Western Blot Analyses of G377::mCherry Secretion—Supernatants were supplemented with protease inhibitors (Roche, Mannheim, Germany) and pellets were lysed in 60 μl RIPA buffer. Detection of respective proteins by Western blot was performed using the rabbit anti-mcherry antibody (Abcam, Cambridge, UK 1/5000).

Immunofluorescence Staining of Gametocytes—After fixation, cells were permeabilized with 0.5% TritonX for 10 min. For visualization of the red blood cell membrane cells were incubated with anti-Ter119::Alexa488 (Biolegend, Fell, Germany, 0.5 mg/ml; 1/1000) antibody for 1h, washed twice with PBS, and resuspended in PBS containing Hoechst for observation under the microscope. Images were either taken on a Zeiss 200 M Axiovert widefield (63x) or Nikon spinning disc (100×) microscope. Image processing was performed with ImageJ.

Mass-spectrometric Analyses of the Gametocyte Egressome—*Experimental Design and Statistical Rationale*—Cells ($n = 3$ biological replicates) were purified via Nycodenz cushion and activated as described above. Mass spectrometric analysis was performed at the CellNetworks Core Facility for Mass Spectrometry and Proteomics of the ZMBH (Zentrum für Molekularbiologie der Universität Heidelberg).

Samples were prefractionated on a 4–15% SDS-PAGE, cut into three slices, reduced with DTT, alkylated with iodoacetamide, and digested with trypsin (Thermo Fisher Scientific, Dreieich, Germany) using a Digest pro MS liquid handling system (Intavis AG, Tübingen, Germany). Digested peptides were then extracted from the gel pieces with 50% acetonitrile/0.1% TFA, concentrated nearly to dryness in a SpeedVac vacuum centrifuge and diluted to a total volume of 30 μl with 0.1% TFA. Ten microliters of the sample was analyzed by a nanoHPLC system (Thermo Fisher Scientific) coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Sample was loaded on a C18 Acclaim PepMap100 trap-column (Thermo Fisher Scientific) with a flow rate of 30 μl/min 0.1% TFA. Peptides were eluted and separated on a C18 Acclaim PepMap RSLC analytical column (75 μm × 250 mm) with a flow rate of 300 nl/min in a 50 min gradient of 3% buffer A (0.1% formic acid) to 40% buffer B (0.1% formic acid, acetonitrile. MS data were acquired with an automatic switch between a full scan and up to ten data-dependent MS/MS scans.

Tandem mass spectra were extracted by Mascot Daemon without grouping or smoothing and analyzed using Mascot (Matrix Science; version 2.4.1). Mascot was set up to search against a *Plasmodium berghei* (strain ANKA) database (obtained through the plasmodb.org database (Plasmodium_bergh_2_11_2014, 4907 entries)) allowing for up to 2 missed cleavages. Trypsin was used as protease, fragment ion mass tolerance was 0.50 Da and a parent ion tolerance of 20 ppm. Carbamidomethylation of cysteine was specified as a fixed modification, deamidation of asparagine and glutamine and oxidation of methionine as variable modifications. Scaffold (version Scaffold_4.0.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (14).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD003487

BioID—10 million blood stage parasites were injected i.p. into a naïve NMRI mouse and the drinking water was immediately supplemented with biotin (SIGMA, Taufkirchen, Germany) at a final concentration of 1 mM. Four days post-infection mice were sacrificed, the blood harvested by cardiac puncture and gametocytes purified by 49% Nycodenz cushion. A small fraction was used for IFA analysis performed with the following antibodies: anti-myc antibody (Roche 0.4 mg/ml, 1/1000) followed by incubation with goat anti mouse Alexa488 (Invitrogen, Dreieich, Germany 2 mg/ml, 1/1000), atto 594-Streptavidin (Sigma, 1/500) or mouse anti-HSP70. Images were either taken on a Nikon spinning disc (100×) microscope or Zeiss Axiovert200. Image processing was performed with ImageJ. For mass-spectrometric analysis *peg3::bira** parasites ($n = 3$ biological replicates) were grown in the presence of biotin and purified by Nycodenz cushion; controls were *peg3::bira** cells grown in the absence of biotin ($n = 3$ biological replicates) and wildtype cells grown in the presence of biotin. Nycodenz purified gametcoytes were lysed with RIPA buffer (50 mM Tris pH8, 1% Nonidet P-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA). 20% of the lysate was kept as input control and biotinylated proteins were enriched using streptavidin coated beads at 4 °C overnight as described (15). Elution of proteins was done with a buffer containing 30 mM of Biotin, 2% SDS, 160 mM NaCl and 6 M Urea followed by mass-spectrometric analysis as described above.

RESULTS AND DISCUSSION

Activation of Gametocytes and Establishing Secretion Conditions—In order to detail the gametocyte egress and osmiophilic body secretion processes, we generated the parasite line *g377::mcherry* expressing a carboxy-terminal mCherry tagged allele of the osmiophilic body protein G377 (PBANKA_1463000) in this haploid protozoan (Fig. 1*A*; supplemental Fig. S1). Initially identified and characterized in *P. falciparum*, the protein is conserved across the genus *Plasmodium* (16, 17) (supplemental Fig. S2). Like in *P. falciparum* (18), mCherry-tagged G377 (G377::mCherry) is trafficked to the periphery of the cell during *P. berghei* gametogenesis (Fig. 1*B*) consistent with a secretion event recently identified for this protein (9).

In order to test the efficiency of recovery of secreted proteins we next collected gametocytes and transferred them into ookinete medium which induces the secretion of osmiophilic body contents, ultimately leading to the formation of mature, free gametes. After 25 min cells were separated from supernatants by centrifugation and both intact cells and supernatants were analyzed by Western blot analysis for G377::mCherry; this assay revealed G377::mCherry in the supernatant only after activation in inducing medium, showing tight control of the egress process by environmental conditions and thus preventing the release of free gametocytes or gametes into the circulatory system of the host (Fig. 1*C*). Non-activated cells treated with saponin to dissolve the RBCM did not show any signal for G377 whereas HSP70—a protein exported to the RBC (19)—was clearly detectable.

Mass Spectrometric Analyses of the Released Egressome—To identify proteins released into the extracellular medium during PVM and RBC lysis, we performed a mass spectrometric analysis of supernatants following gametogenesis; this gamete egress secretome is called egressome hereafter. We first separated red blood cells infected with gametocytes from those infected with asexual stage parasites or noninfected ones by Nycodenz density gradient centrifugation; purified gametocyte preparations isolated from one infected mouse were then activated in ookinete medium for 25 min at 19 °C followed by separation of whole cells from secretion supernatants by centrifugation (Fig. 1*D*). These supernatants were finally separated by SDS-PAGE and processed for mass spectrometric analysis.

In three independent experiments, we identified a total of 86 proteins in at least two of them (supplemental Table S1). Among the proteins known to be involved in egress, G377 appeared as the most abundant with a sum total of 317 peptide hits, MDV1/PEG3 with 30, GEST with 20 and PPLP2 with 20 hits.

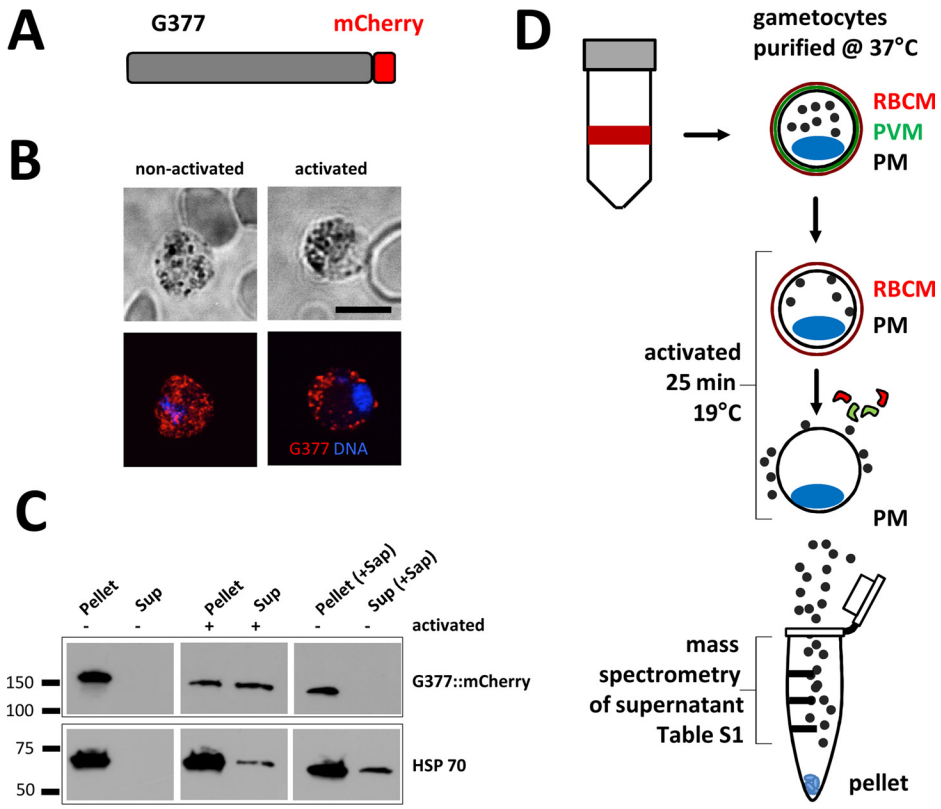

FIG. 1. ***A*, G377::mCherry model.** *B*, Imaging of G377::mCherry parasites before (non-activated) and after activation of gametogenesis. In females G377 re-localizes to the parasite periphery upon activation. Scale bar: 5 μm. *C*, Western blot of the G377::mCherry secretion assay approach. G377::mCherry cannot be detected in supernatants of unactivated gametocytes but results in a clear band upon secretion in activated cells. *D*, Schematic of biochemical egressome analyses from purification of non-activated gametocytes to the collection of secreted proteins.

A linear regression analysis between the egressome and the global proteome of whole, non-activated gametocytes (20) showed no correlation ($r = 0.1819$) with protein abundance. Eighty-four of the 86 identified proteins had been identified previously in purified males or females (71 proteins), or mixed gametocytes (13 proteins) of *P. berghei* (20).

The Egressome is Enriched in Putatively Secreted Proteins—Forty-three of the 86 proteins of the egressome contain a predicted N-terminal signal peptide. The enrichment for such factors (hypergeometric tests, $p = 7.3e\text{-}12$) suggested that hitherto unknown proteins had been identified that are secreted during gamete egress and perhaps aid the dissolution of PVM and RBCM.

We identified the serine proteases subtilisin (SUB) 1 and SUB2, as well as the uncharacterized aspartate protease PBANKA_144900; none have been implicated in gamete egress before although both SUB1 and SUB2 are key to merozoite egress and subsequent invasion of the red blood cell. SUB1 promotes merozoite PVM dissolution and activates SERA papain-like cysteine proteases (21, 22). Intriguingly we identified three SERA proteases in the egressome (supplemental Table S1). SUB2 is processed at the N terminus to yield a fully functional protease and is required for the shedding of AMA-1 during RBC invasion (23, 24), however whether it plays a role during merozoite egress is unknown. Additional and putatively exported proteins upon activation include PSOP1 and PSOP12, MTRAP, and three ETRAMP proteins, Fam-a, and the two exported, unique *Plasmodium* proteins PBANKA_030060 and PBANKA_062310.

Likely Extracellular PV and RBC Contaminants—Belonging to a group of most likely extracellular PV-, PVM- or RBC-resident factors are the translocon components HSP101 and PTEX150 (25), the parasitophorous vacuole protein PV1 (26) and IBIS1 from Maurer's cleft-like organelles (27). IBIS1 does not contain a signal peptide but is transported to the RBC virtue of its PEXEL motif. Three additional proteins are annotated as such: PBANKA_140070 (PEXEL), PBANKA_070070 (PEXEL) and PBANKA_122900 (PEXEL). It is likely that they were already present in the gametocyte-infected RBC and were thus detected in our analysis.

Likely Intracellular Contaminants from Males—Ribosomal proteins are among the most abundant proteins in the female gametocyte (20). With the exception of S2B the complete lack of ribosomal proteins in the egressome shows that whole, female gametes did not contaminate the mass-spectrometry sample. Likewise, proteins of the axoneme and flagella of the mature male were not detected at all, although they are among the most abundant proteins detected exclusively in

the male proteome (20). They include for example three different dyneins PBANKA_050730, PBANKA_041610, and PBANKA_092540. The remaining proteins that we detected originate thus most likely from male gametocyte remnants following the successful release of flagellated males. They include: plasmepsin X (a protease related to hemoglobin-degrading proteins); the food (digestive) vacuole residents M1-family aminopeptidase, dipeptidyl aminopeptidase and bergheilysin (28); nine glycolytic enzymes; three protein translation-associated proteins (EF1alpha, eIF4A, 40S ribosomal protein S2B); four cytoskeletal proteins (alpha and beta tubulin, actin I and II); and nine DNA/RNA binding and modifying proteins.

*Tagging of OB Protein MDV1/PEG3 with BirA**—In order to identify which proteins are specifically present in osmiophilic bodies we adapted the BioID methodology (15) for the use of *in vivo* labeling of proteins of *P. berghei* gametocytes present in circulating red blood cells of the mouse host. BioID relies on the fusion of a protein of interest with a mutated BirA ligase (BirA*) that in the presence of excess biotin biotinylates nearby proteins, which can be purified by affinity chromatography using streptavidin. MDV1/PEG3 or PBANKA_143220 is a 214 amino acid long protein with a molecular weight of 24 kDa located in the OB (8, 29) and detected in the above described egressome of mixed gametocytes. The protein has been identified in male as well as female gametocytes (8, 9, 20) and is thus a better BirA*-tagging candidate than G377, which is specifically expressed in females. Like G377, MDV1/PEG3 is also conserved in *Plasmodium spp.* (supplemental Fig. S3). We tagged the endogenous MDV1/PEG3 protein at the carboxy-terminus with the mutated, promiscuous *E. coli* BirA* protein and a triple c-myc tag (Fig. 2*A*). Transgenic *mdv1/peg3::bira** parasites were generated via single crossover of a plasmid construct leaving the tagged gene under the control of its own endogenous promoter in this haploid protozoan (supplemental Fig. S4).

First we tested the biotinylation procedure by IFA. Mutants grown in the presence or absence of biotin were analyzed by immunofluorescence. Using an anti-myc antibody the fusion protein is readily identified by immunofluorescence revealing its gametocyte-specific localization in the mutant with an OB-typical, abundant localization (Fig. 2*B*) identified for G377, GEST, and MDV1/PEG3 (7–9) (please compare also with Fig. 3*A* for localization of G377, MDV1/PEG3, and GEST). In addition, we stained the cells with fluorescently-labeled streptavidin; only parasites grown in the presence of biotin showed strong labeling of the entire gametocyte and corresponding to the c-myc staining resulting in bright yellow signal. As an additional control we assayed wildtype cells grown in the presence of 1 mM biotin; neither anti-myc nor streptavidin produced appreciable staining in these cells. The immunofluorescence assay thus showed that the biotin labeling procedure is specific for the presence of MDV1/PEG3::BirA* and in need of excess biotin.

MDV1/PEG3::BirA Biotinylation Requires Excess Biotin*—Following the immunolabeling analyses of the *mdv1/peg3::bira** mutant we determined the presence of biotinylated proteins in gametocyte lysates by Western blot analysis. 10 million *mdv1/peg3::bira** or wildtype blood stage parasites were injected i.p. into a naïve NMRI mouse and the drinking water was immediately supplemented with biotin in tap water at a final concentration of 1 mM. As a control for specific biotinylation *mdv1/peg3::bira** parasites were grown in the absence of biotin. Four days postinfection the blood was harvested by cardiac puncture and gametocytes purified by Nycodenz gradient centrifugation. Biotinylated proteins were affinity purified on a streptavidin column at 4 °C (Fig. 2*C*).

Using a streptavidin-HRP conjugate we detected a single band in the presence of biotin that in size is consistent with the *mdv1/peg3::bira** fusion protein; neither wildtype grown in the presence of biotin nor *mdv1/peg3::bira** cells grown in the absence of biotin produced a signal. The same-sized band was detected in both samples expressing MDV1/PEG3::BirA* when probing with the c-myc antiserum. *Plasmodium* HSP70 was used as a loading control and could be detected in all three samples (Fig. 2*D*).

Western blot analysis with anti-myc revealed equally-sized bands in the *mdv1/peg3::bira** lines grown in the presence or absence of excess biotin, but only the presence of biotin resulted in a strong signal at the same molecular weight as anti-myc detected protein. The absence of strong streptavidin signal in the remainder of the blot suggested that predominantly MDV1/PEG3::BirA* had been auto-biotinylated. As expected, wild-type lysates, probed with either anti-myc or streptavidin, did not produce any signal.

Mass Spectrometric Identification of the Osmiophilic Body Proteome Through Biotinylation by MDV1/PEG3 Tagged with BirA and Streptavidin Affinity Purification*—We identified biotinylated proteins from mice supplied with and without biotin, as well as from a mouse infected with a wildtype line and supplied with the same amount of biotin. Mass-spectrometric analyses of three independent experiments resulted in the identification of a total of 34 proteins via the BioID approach (supplemental Table S2). Among those, 22 (65%) had also been detected in our egressome data set. These included the known egress proteins MDV1/PEG3, G377, and PPLP2 but not GEST.

Twenty out of the 34 identified proteins are predicted to bear an amino-terminal signal peptide. None of the novel proteins detected has previously been implicated in gamete egress, although 12 gene deletion mutants had been analyzed in the context of parasite transmission (see www.pberghei.eu, (30)). Δ*psop1* and Δ*psop12* parasites for example showed a 51 and 32% reduction in oocyst numbers (31). PSOP12 belongs to the 6-cys protein family of Plasmodium and was detected together with three other family members: P230, P230p, and P47 (32). P47 is a female gametocyte-specific protein displayed on the surface of the gamete and required for fertilization; *in vitro* fertilization rates of knock out mutants are reduced to less than 1% of wildtype. P230 and P230p on

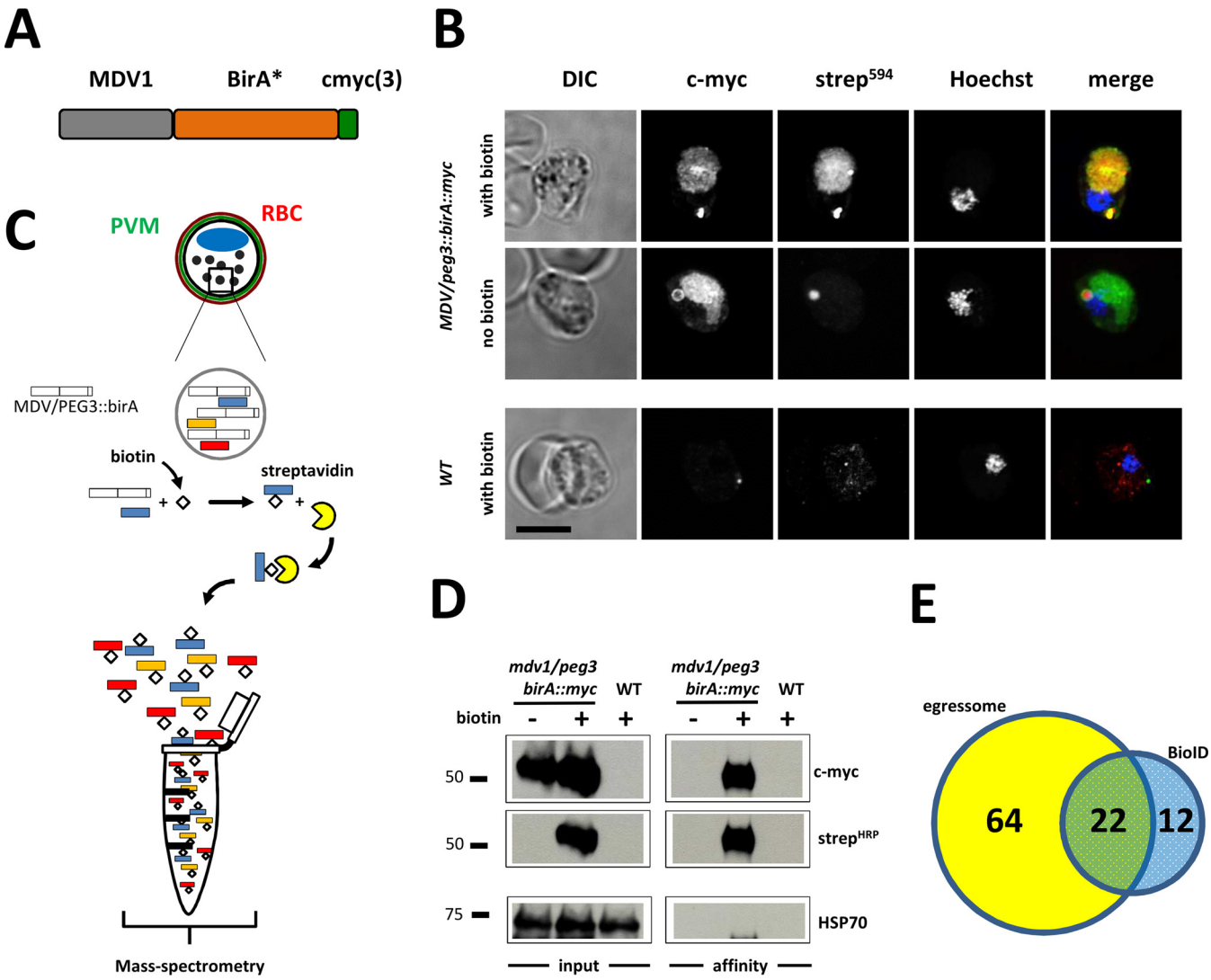

FIG. 2. ***A*, MDV1/PEG3::BirA*::myc model.** *B*, IFA of MDV1/PEG3::BirA* and wildtype gametocytes in the presence or absence of biotin stained with anti-myc, streptavidin 594 and Hoechst. Scale bar: 5 μm. *C*, Schematic of the BioID approach and streptavidin affinity purification. *D*, Western blot of MDV1/PEG3::BirA* and wildtype gametocyte lysates before and after affinity purification probed for anti-myc, streptavidin HRP and HSP70. *E*, Venn diagram of proteins detected upon activation of gametocytes and BioID approach. 86 proteins were detected in the egressome, 34 in the BioID approach.

the other hand are restricted to males. Δ*p230* fertilization rates were as low as in the *p47* knockout mutant, whereas Δ*p230p* males fertilization was in the normal range. A surprising find was the presence of three female-specific LCCL domain-containing proteins (33): CCp1/LAP2 (female proteome), CCp3/PbSR/LAP1 (female proteome) and CCp5/LAP3 (female proteome/male proteome 1 single hit). There are six LCCL domain containing proteins but the other three members are translationally repressed and thus only translated in the ookinete (34). LAP2 and LAP3 have been shown to be expressed in the gametocyte whereas in the ookinete they label the crystalloid organelle in *P. berghei* (35). None of the LAP proteins itself are essential for gametogenesis or ookinete formation in *P. berghei*, but rather affect sporozoite formation in oocysts (36, 37).

We recovered the aspartyl protease PBANKA_144900 and subtilisin 2 (SUB2), albeit once in a control data set. Both have a predicted signal peptide and a C-terminal transmembrane domain suggesting that they could be retained at the parasite surface after secretion. Although the former is still uncharacterized, SUB2 is a micronemal protease released onto the merozoite surface during where it facilitates cleavage/shedding of AMA-1 during erythrocyte invasion and an essential protein for blood stage development (23, 24).

We identified a number of proteins that are most likely biotinylated during trafficking and folding of MDV1/PEG3::BirA* within the secretory pathway; they are the chaperones endoplasmin (GRP94), HSP70 (PBANKA_081890, GRP78, BIP), HSP70 (PBANKA_071190) and HSP110, as well as the calcium-binding protein ERC and a unique conserved Plas-

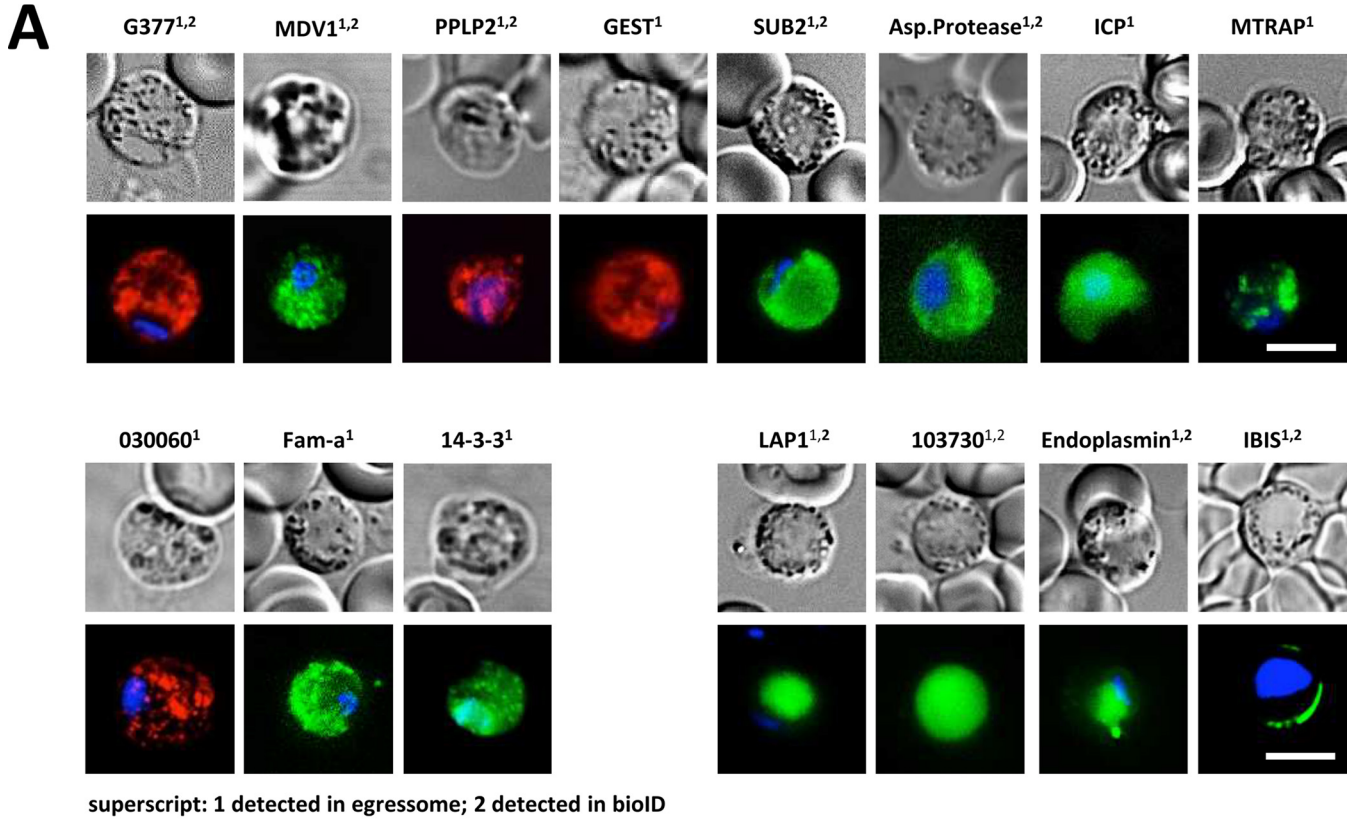

FIG. 3. ***A,* Live cell imaging of parasites expressing candidate proteins fused to either GFP or mCherry.** Superscript numbers indicate whether the protein was detected in the egressome (1) and/or with BioID (2). Scale bar: 5 μm. *B,* Protein models to illustrate the divergent domains existing in the identified proteins.

modium protein (PBANKA_103730) with the carboxy-terminal ER retention signal SDEL.

GFP-tagging Confirms the Presence of Novel Egress Molecules in Sexual Precursor Cells with a Secretory Vesicle-like Localization—In order to investigate the presence of a subset of these newly identified factors in secretory vesicles of gametocytes, we next tagged a selection of proteins identified in the egressome and/or bioID approach with a carboxy-terminal fluorescent tag (GFP or mCherry). Each protein was targeted for *in situ* tagging of the endogenous locus with a standard plasmid vector that linearized to facilitate integration by homologous recombination into the endogenous locus;

genotyping by PCR was used to verify the integration event (supplemental Fig. S5).

In this study we tagged a total of 15 proteins (Fig. 3*A*). G377, MDV1/PEG3, GEST, and PPLP2 produced staining patterns consistent with OB or secretory vesicle localization. The fluorescent protein tagging resulted in the first tagging of G377 and PPLP2 in *P. berghei*, which localized similar to their orthologous proteins in *P. falciparum* and confirmed the presence of MDV1/PEG3 (8, 9) and GEST (7) in vesicular structures.

The staining patterns of seven new proteins are reminiscent of egress vesicle localization (Fig. 3*A*). They are SUB2, the aspartyl protease PBANKA_144900, MTRAP, PBANKA_030060, Fam-a, and 14-3-3 even though their pattern is not always as clearly structured. ICP, LAP1, endoplasmin and PBANKA_103730 on the other hand produced a more homogenous staining within the gametocyte. IBIS1 was clearly localized outside the gametocyte (Fig. 3*A*). These localization data suggest that these proteins are not at all, or not exclusively located in OBs.

Apart from containing N-terminal signal peptide sequences (nine out of eleven), the proteins already known to take part in egress of gametocytes and those identified here to present with a secretory vesicle localization share no sequence homologies (Fig. 3*B*). Egress thus involves a plethora of factors, whose interactions and molecular functions are largely unknown.

Validation of Selected Proteins Using Gene Deletion—From the proteins shown to localize in gametocytes we chose PBANKA_030060 (a protein with a highly repetitive internal amino acid sequence; Fig. 3*B*), aspartyl protease PBANKA_144900 and MTRAP to be characterized as gene deletion mutants. Gene knockouts (Δ*pbanka_030060*, Δ*pbanka_144900*, Δ*mtrap*) were generated by transfection of linearized plasmid vectors that replaced the endogenous gene in this haploid eukaryote with a pyrimethamine resistance marker. Genotyping by PCR was used to verify the integration event and clonal lines established by limiting dilution (supplemental Figs. S6, S7, S8).

In parallel with wildtype parasites, each mutant was quantified in a standard ookinete formation assay and the egress behavior was assayed by DIC and immunofluorescence against the RBC marker Ter119. Gametocyte egress, highlighted with anti-Ter119 staining of the RBC, was similar to wildtype for Δ*pbanka_030060* and Δ*pbanka_144900* (Fig. 4*A*–4*C*) and ookinete formation proceeded also as wildtype controls with an ookinete conversion rate around 50% (Fig. 4*E*). Ookinetes from the wildtype and both gene deletion mutants moved at normal speed (Fig. 4*F*). Therefore, both proteins appear to be non-essential for sexual development and ookinete formation. In accordance with our findings are previous studies using protease inhibitors that showed the inhibition of aspartyl proteases with EPNP did not result in a significant change in gamete egress, whereas cysteine/serine protease inhibitors TLCK and TPCK, the cysteine protease inhibitor E-64d, as well as the serine protease inhibitor PMSF resulted in reduced egress (38).

In contrast, MTRAP emerged as a crucial factor for gamete egress. Δ*mtrap* parasites did not form ookinetes after *in vitro* overnight culture ($n = 4$). IFAs of activated gametocytes with the anti-Ter119 antibody showed a clear defect in egress of males and females (Fig. 4D). Males presented deformed red blood cells and live microscopy further showed a characteristic exflagellation phenotype; in contrast to wild-type cells (Video S1) Δ*mtrap* males are trapped within the RBC yet motile (Video S2). Affecting both male and female gamete egress, Δ*mtrap* parasites failed to produce ookinetes ($n = 4$) in a standard overnight *in vitro* culture (Fig. 4*E*). Consistent with the egress phenotype, infection of mosquitos did not result in the formation of oocysts ($n = 3$). Experiments were performed with 2 Δ*mtrap* parasite clones obtained from two independent transfections. Thus, only one of the three candidate proteins tested turned out to have an essential function. Egress most likely requires the concerted action of multiple proteins that itself may have redundant functions. Gene deletions of G377, MDV1/PEG3, GEST, and PPLP2 have also been shown to produce minor to severe reductions but never an absolute block in parasite transmission.

CONCLUSIONS

Using mass spectrometric analysis, we established a *P. berghei* gamete egressome. In total we could detect 86 proteins in the egressome, 41 proteins with a predicted N-terminal signal peptide were found, which are thus potentially secreted molecules and implicated in the egress process. These may contribute or be key to the lysis of the PVM and RBCM. We also confirmed G377 as a reliable marker of intracellular OB in *P. berghei* as well as a marker for OB secretion.

Along with the endogenous C-terminal tagging of selected proteins with GFP or mCherry we have identified MTRAP as a novel factor essential for gametocyte egress from the red blood cell; the protein is key for parasite transmission into the mosquito. MTRAP belongs to the TRAP family of proteins thought to exclusively play distinct roles during parasite adhesion, invasion, and motility. MTRAP was originally found to function in merozoite invasion of the erythrocyte (39, 40). However recent results rather suggest a role in signaling events necessary during that process (41). Using the rodent malaria model *P. berghei*—better accessible to gene modifications than *P. falciparum*—our data show that MTRAP is redundant for blood stage development of the parasite (essential proteins cannot be deleted) but crucial for parasite transmission by playing a key role for gamete egress from the RBC. Yet, the precise role of the protein during gametogenesis still needs to be addressed. Previous attempts to knockout MTRAP in *P. falciparum* were not successful (40).

Although the defect of the *mtrap* knockout is clear, previous gene deletion mutants of *gest* or *g377* for example produced

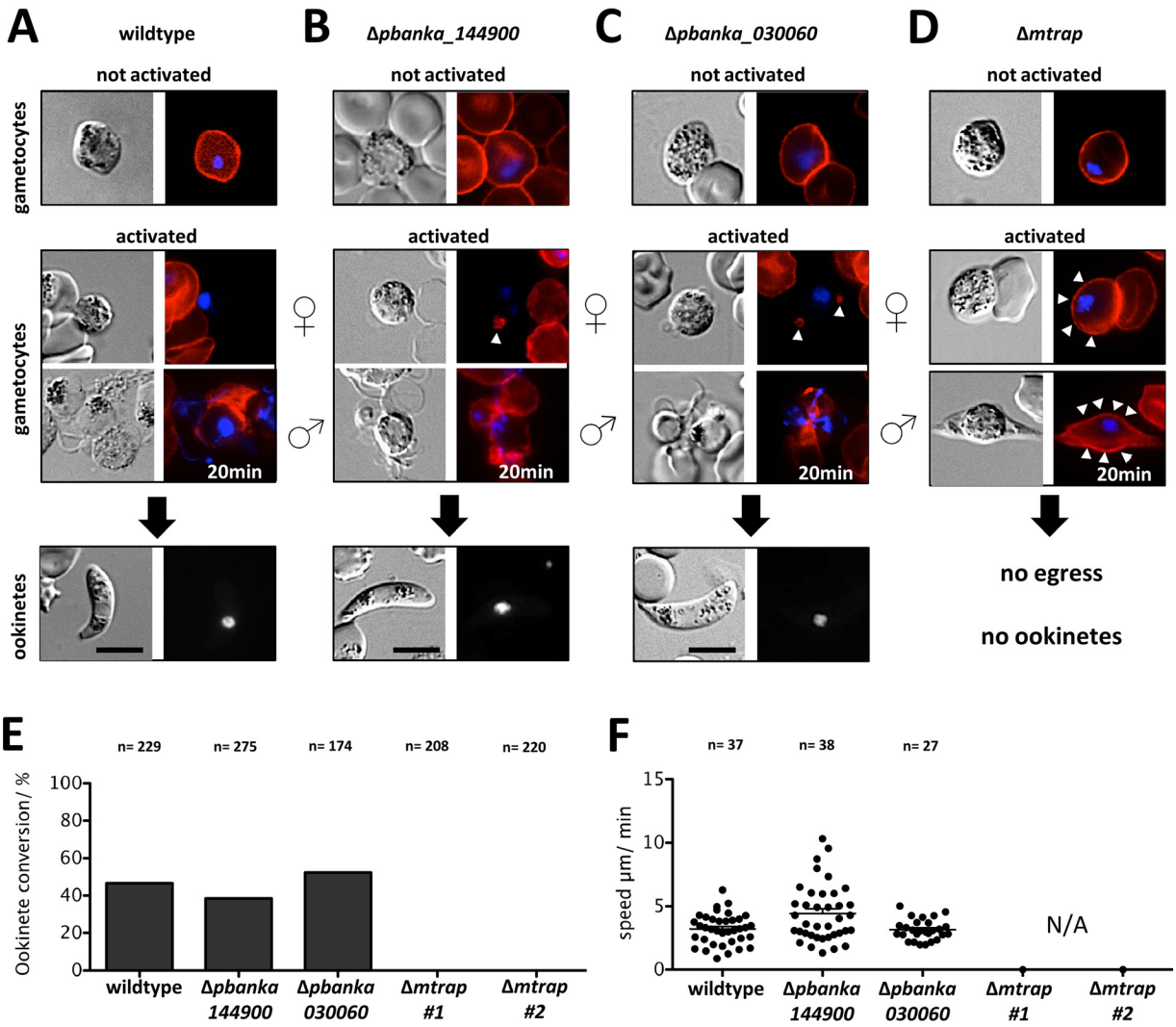

FIG. 4. ***A–D,*** **IFA of gametocytes before and 20 min post activation of gametogenesis.** RBCM (red) is stained with anti-Ter119 and the nucleus (blue) with Hoechst. Wildtype cells **(***A*), Δ*pbanka_144900* (*B*) and Δ *pbanka_030060* (*C*) gametes egress normally and produce ookinetes, whereas two independent clones of MTRAP knockout mutants (*D*) fail to produce free gametes and remain trapped inside the RBCM. Scale bar: 5 μm. *E,* Ookinete conversion rates of wildtype and knockout mutants. *F,* Ookinete speed of wildtype and mutants.

milder defects in egress and transmission; the process of gametogenesis is thus likely a multifactorial one, requiring proteins acting in concert or within a yet to be established cascade. Although gametocytogenesis is markedly slower in *P. falciparum* and produces elongate sexual precursors, the egress of mature gametes has been shown to rely on orthologous protein factors—G377 and PPLP2 for example—in both the human and rodent malaria parasites. Therefore *P. berghei* is an appropriate model to study this process, especially because the parasite is easily manipulated genetically, and established methodologies are at hand to evaluate egress, fertilization, and ookinete formation.

Our study identifies a number of proteins known to function in merozoite egress: *e.g.* SUB 1 and cystein protein like sera protease. SUB 1 is secreted from exonemes. Perhaps OB and related egress vesicles are functionally related to these or even micronemes. In *T. gondii* micronemes secrete PPLP1 in order to disrupt the host cell for egress (42). Both, merozoite and gamete egress are dependent on calcium signaling (43) and involve a DOC2 protein (44). In the gamete this cascade most likely relays the environmental change to the intracellular parasites; yet what triggers merozoite egress is unknown.

In summary we present two different mass spectrometric approaches to identify proteins involved in gametocyte egress fundamental for life cycle progression. Using GFP/mCherry tagging of selected candidates together with gene deletion we identify MTRAP as a novel essential factor involved in that process. The establishment of a vesicular BioID approach should be a means to identify exoneme proteins in the merozoite and could also be applied to ookinetes and sporozoites.

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§ To whom correspondence should be addressed: University of Heidelberg Medical School, Im Neuenheimer Feld 345, Heidelberg 69121 Germany. Tel.: 06221-567438; E-mail: freddy.frischknecht@med.uni-heidelberg.de; gunnarmair@yahoo.com.

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